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Award Number: DAMD17-00-1-0279

TITLE: Kinase Independent Functions of Cyclin D1 Which Contribute to its Oncogenic Potential In Vivo

PRINCIPAL INVESTIGATOR: Mark W. Landis
Philip W. Hinds, Ph.D.

CONTRACTING ORGANIZATION: Harvard Medical School
Boston, Massachusetts 02115

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual Summary (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Kinase Independent Functions of Cyclin D1 Which Contribute to its Oncogenic Potential In Vivo

5. FUNDING NUMBERS
DAMD17-00-1-0279

6. AUTHOR(S)
Mark W. Landis
Philip W. Hinds, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Harvard Medical School
Boston, Massachusetts 02115
E-Mail: mlandis@fas.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)

Cyclin D1, an important cell cycle regulator, is a potent oncogene in several tumor types, including breast cancer. The most well understood function of cyclin D1 is to bind and activate cdks 4 and 6. One target of these kinases is pRb. Upon phosphorylation, pRb is inactivated, and cells pass from G1 into S phase. We and others have demonstrated that cyclin D1 has other functions, many of which are independent of kinase activity *in vitro*. *In vivo* demonstration of kinase independent functions of cyclin D1 may help elucidate the underlying mechanisms of cyclin D1 oncogenicity.

To determine whether cyclin D1 has important kinase-independent functions *in vivo*, we are generating a cyclin D1 K112E knock-in mouse. This single base change results in a cyclin that can bind to, but not activate the kinase partner. As the locus will be left almost undisturbed, we expect that the mutant allele will be expressed in a normal manner. The phenotype of the mouse will be analyzed to determine whether any of the phenotypes of the cyclin D1 -/- mouse are rescued. This analysis will allow dissection of how the kinase-independent functions of cyclin D1 contribute to development, proliferation and oncogenesis *in vivo*.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)	15. NUMBER OF PAGES
Cell Cycle, cyclin, development, animal model, proliferation control oncogene, tumor suppressor, knock-in mouse	9
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Cyclin D1, an important cell cycle regulator, is a potent oncogene in several tumor types, including breast cancer. The most well understood function of cyclin D1 is to bind and activate cdks 4 and 6. One target of these kinases is pRb. Upon phosphorylation, pRb is inactivated, and cells pass from G1 into S phase. We and others have demonstrated that cyclin D1 has other functions, many of which are independent of kinase activity *in vitro*. *In vivo* demonstration of kinase independent functions of cyclin D1 may help elucidate the underlying mechanisms of cyclin D1 oncogenicity.

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Body

To determine which functions of cyclin D1 are important *in vivo*, we have undertaken the generation of a knock-in mouse. A targeting construct has been generated (figure 1) and electroporated into mouse embryonic stem (ES) cells. Following selection by G418, clones were selected and screened by several methods including Southern Blot, PCR and karyotype. As demonstrated, several clones screened positive for homologous recombination at the 3' end by Southern (figure 2). These clones were then screened for homologous recombination from the 5' end and found to be correct. Clones that tested positive for homologous recombination from both ends were then subjected to PCR to ensure that a restriction site closely linked to the point mutation was retained (figure 3). Clones that were positive for this test were then subjected to karyotyping to ensure the proper number of chromosomes were present prior to injecting into blastocysts. Six cell lines were found that met all of the criteria and one was chosen for injection.

From the injection, three male and one female chimeras were obtained. Two of the males produced a total of seven agouti offspring, indicating germ line transmission of the ES cells. Of the seven offspring, three males screened positive for the KE allele by PCR (figure 4). These males were then crossed to wild-type animals to generate female animals carrying the allele.

Key Research Accomplishments

- Generation and Screening of ES clones resulting in Six clones suitable for injection
- Generation of chimeric mice by blastocyst injection
- Observation of germ line transmission of the K112E allele generating heterozygotes

Reportable Outcomes

- Generation of ES cell lines transgenic for the K112E allele
- Generation of heterozygous animals carrying the K112E allele
- Acceptance to the American Association of Cancer Research "Pathobiology of Cancer" Workshop

Conclusions

Several critical steps on the way to generating the homozygouse knock-in mouse have been accomplished. ES cell clones have been generated and used to create heterozygous animals. We are now only a couple of steps from completing the generation of these animals. We are in the process of mating the heterozygotes to a nestin-Cre deleter strain to remove the selection cassette and will then mate the animals to homozygosity. We will then undertake the characterization of these animals closely. This work has become even more relevant considering the recent report that the absence of cyclin D1 can completely protect mice against some induced breast cancers (1). The project currently underway should allow the determination of whether the lack of cyclin D1 directed kinase activity is sufficient to protect against breast cancer or if the oncogenic activities of cyclin D1 are kinase-independent. This knowledge will allow better determination of how cyclin D1 might serve as a target for therapeutic intervention in breast cancers.

References

1. Yu Q. Geng Y. Sicinski P. (2001). Specific protection against breast cancers by cyclin D1 ablation. Nature. 411(6841):1017-21.

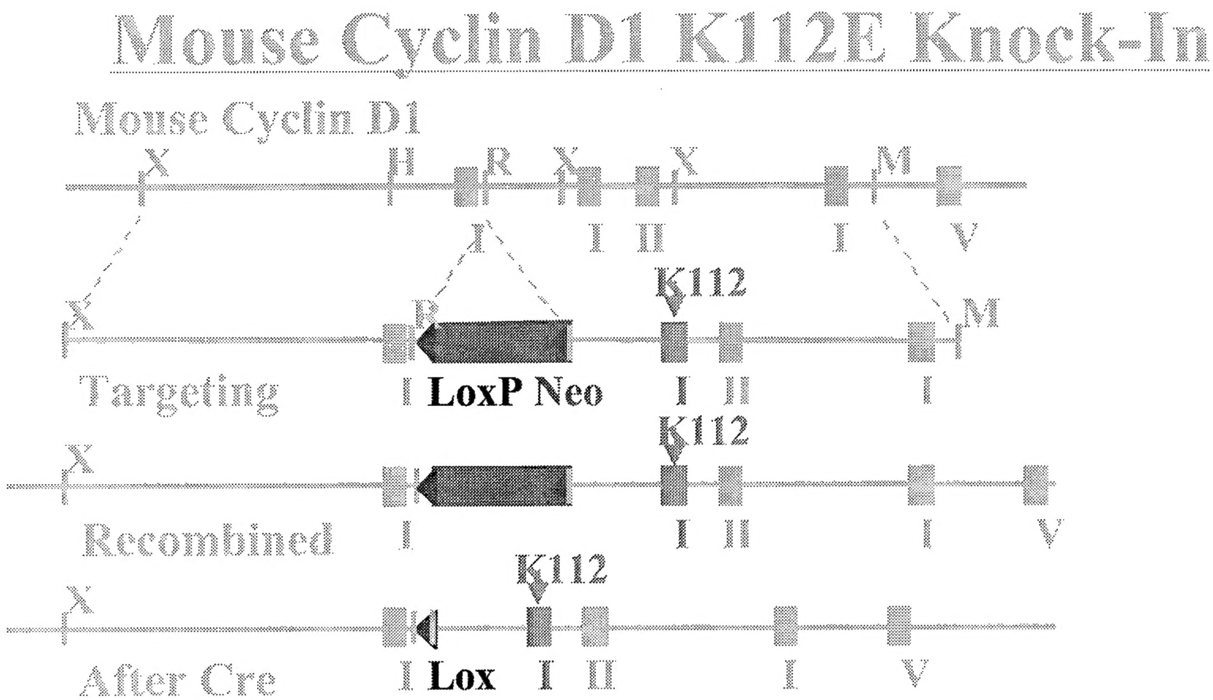

Figure 1. The targeting cassette used to generate ES cells contains a floxed-neo gene used for selection and a single base change that will generate the K112E mutation. After recombination and treatment with the Cre recombinase, the locus will be left virtually undisturbed.

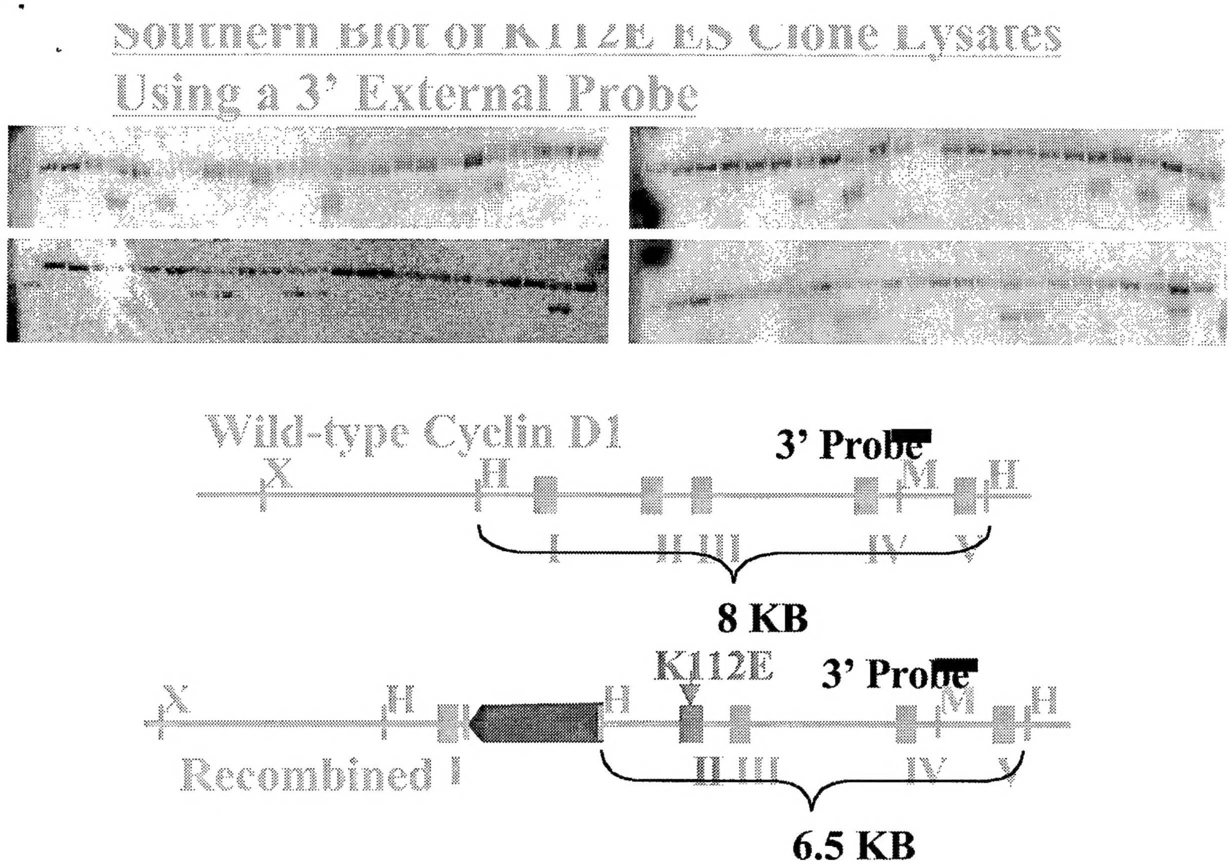

Figure 2. Southern blot of ES cell lysates demonstrates homologous recombination events in some cell lines. Any positive clone will have both a large band and a smaller band corresponding to the knock-in allele.

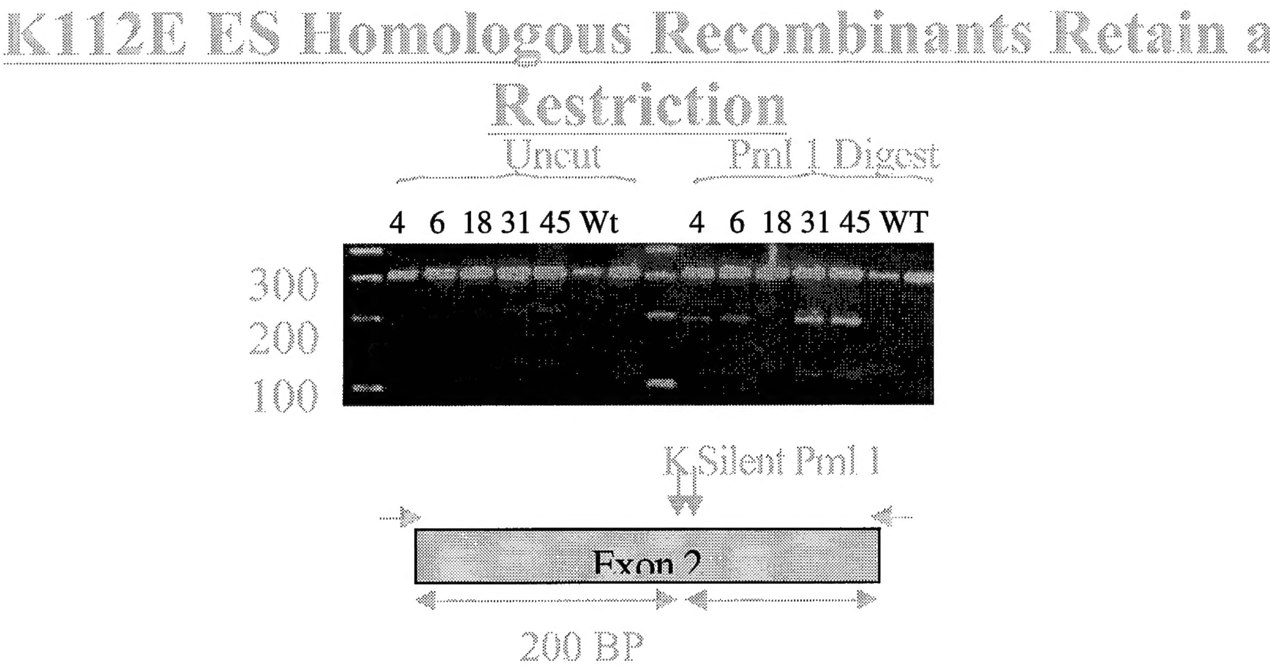

Figure 3. DNA from ES cells was subjected to PCR to determine whether the clones were transgenic for the desired allele. A silent Pml1 site was built in to the targeting cassette. PCR across the region yields a 300 bp fragment that when digested give s a 200 and 100 bp fragment indicative of the knock-in allele. Clones 4, 6, 31 and 45 all demonstrate the knock-in allele, whereas a wild-type (Wt) clone and clone 18 do not show these bands when digested with Pml1.

PCR of 3 F1 Progeny of KE Chimeric Males emonstrates Germ-Line Transmission of the Allele

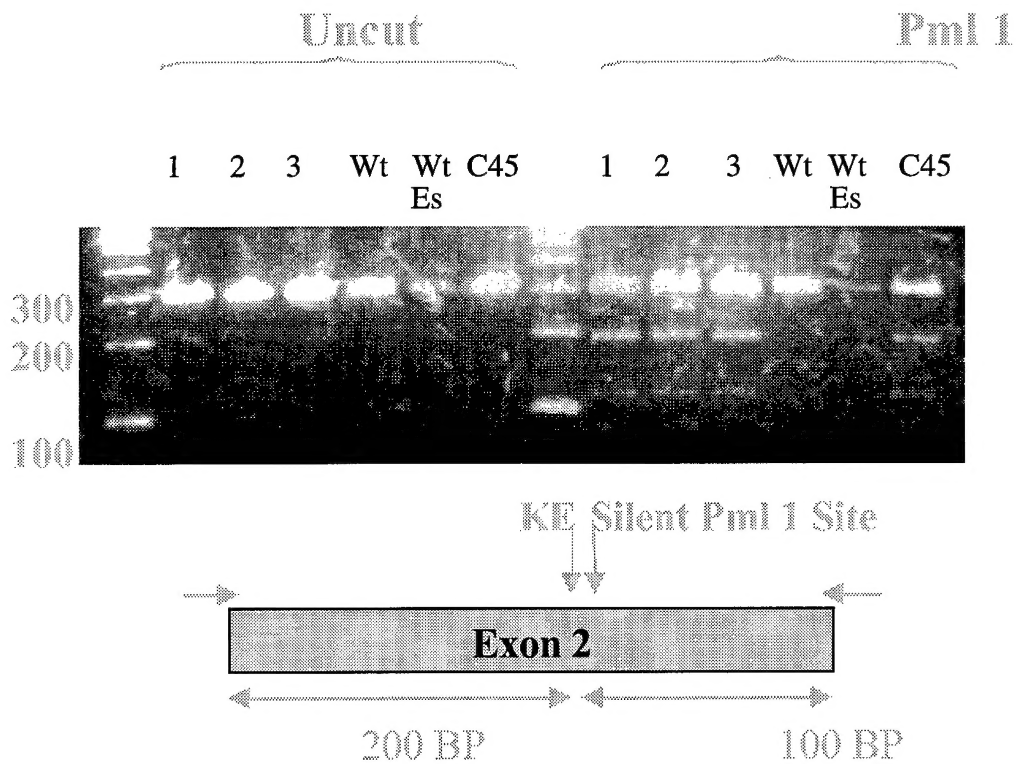

Figure 4. DNA from agouti progeny of the chimeric animals was subjected to PCR to determine whether the animals were transgenic for the desired allele. A silent Pml1 site was built in to the targeting cassette. PCR across the region yields a 300 bp fragment that when digested give s a 200 and 100 bp fragment indicative of the knock-in allele. Agouti progeny 1-3 and C45 (the original ES clone used to generate the chimeras) all demonstrate the knock-in allele, whereas a wild-type (Wt) mouse and wild-type ES cells do not show these bands when digested with Pml1.